Departments of Defense and Agriculture team up to develop new insecticides for mosquito control

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Mosquito-borne pathogens are among the most important sources of human disease that cause morbidity and mortality worldwide. They include the viruses responsible for deadly outbreaks of yellow fever, Rift Valley fever, eastern equine encephalitis, Japanese encephalitis and dengue, and an assortment of other serious illnesses caused by the etiological agents of West Nile fever, St Louis encephalitis, Murray Valley encephalitis, Venezuelan equine encephalitis and chikungunya disease. Dengue viruses, of which there are 4 serotypes, cause an estimated 50-100 million new illnesses each year (and 25,000 deaths) while the latest chikungunya epidemic has lasted longer, affected more people, and occurred over a wider geographic area than any previous outbreak of the disease. Yellow fever outbreaks continue to occur sporadically in South America and Africa when either vaccination or vector control are inadequate. These outbreaks have been controlled by creating barrier zones of vaccinated people and by increasing the intensity of vector control. The threat of devastating outbreaks of yellow fever remains, as illustrated by continuing quarantine and vaccination requirements for international travel. The most devastating of all mosquito-borne diseases is malaria, which kills an estimated 1 million people annually, while infecting another 500 million. Although public health efforts have been able to reduce or eliminate vector-borne pathogens in many situations, some parts of the world have actually suffered increases during the past 30 years. A number of agencies have responded to this problem with much increased levels of attention: World Health Organization, Bill and Melinda Gates Foundation, President's Malaria Initiative, Institute Pasteur, US Centers for Disease Control and Prevention, and US National Institutes of Health. However, morbidity and mortality due to mosquito-borne diseases is increasing.

Today, mosquito wars are being fought around the globe and on many fronts. Insecticide-treated bed nets are mass-produced and distributed to the hardest-hit malarious regions in Africa, India and southern Asia. Vaccines have been developed to protect humans and domestic animals against Yellow fever, Japanese encephalitis, Rift Valley fever and eastern equine encephalitis, with intensive ongoing research targeting dengue, West Nile virus, and malaria vaccine development. New skin and clothing repellents for personal protection against all biting insects are being developed, and insecticide and related application technology development is in full swing. Of these, the key component for protecting humans from mosquito-borne illness is the use of effective insecticides that quickly

Figure 1: Twenty-four-well plate bioassays used to expose first instar *Aedes aegypti* larvae to candidate chemical compounds for insecticide discovery. Five larvae are added to each well and observed for mortality after 24 hours.

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kill millions of mosquitoes before they can pass their pathogens to sicken or kill humans. Mosquito adulticides and larvicides are a key component of our assault, along with indoor residual spraying and insecticide-treated bed nets.

Unfortunately, mosquitoes are fighting back somewhat successfully by developing resistance to currently used mosquito adulticides. To date, at least 100 species of pathogen-carrying mosquitoes have overcome the effects of today's limited arsenal of adulticides. We now have only 2 chemical classes of adulticides available for adult mosquito control: organophosphates (OPs) and pyrethroids. Malathion is one of our oldest organophosphate adulticides and the workhorse of this class. It was developed in the early 1950s for agricultural pest control and has been used extensively around the world as a mosquito adulticide since 1953. It is a cholinesterase inhibitor that impairs nerve cell transmission. Resistant mosquitoes have at least 3 biochemical processes for detoxifying this class of insecticide. Pyrethroid insecticides were developed in the 1970s as analogs of pyrethrum, a natural product of chrysanthemum flowers, known for its insecticidal properties for hundreds of years. Pyrethroids provide rapid knockdown of mosquitoes by binding to sodium channels on nerve cells and subsequently depolarizing them to stop neural transmission. Resistant mosquitoes are now capable of detoxifying pyrethroids by the above 3 biochemical processes and target cell insensitivity. Larvicides offer more target sites for killing immature mosquitoes, but increased tolerance or resistance has also been reported among different larvicide classes including the stomach poison *Bacillus sphaericus*, insect growth regulator

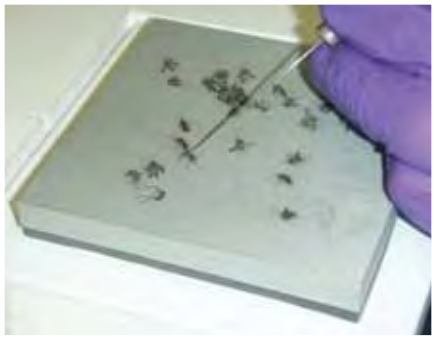

Figure 2: A repeating dispenser used for applying minute amounts of candidate insecticides to the thorax of adult mosquitoes. Mosquitoes are immobilized on a chill table.

(methoprene), and a commonly used OP (temephos) among some mosquito species.

With only 2 adulticide classes left to combat an increasingly resistant adult mosquito population and the pressing need for novel larvicides with new modes of action, a call has been initiated by the United States Department of Defense (DoD) and public health agencies to develop new classes of insecticides that are affordable and efficacious in killing mosquitoes, especially resistant ones. At the United States Department of Agriculture (USDA), several research units within the

Figure 3: Dosed adult mosquitoes are held for 24 hours to determine insecticidal activity.

Agricultural Research Service (ARS) are actively involved in discovering and developing novel insecticides. These units include the Mosquito and Fly Research Unit (MFRU) at the Center for Medical, Agricultural and Veterinary Entomology (CMAVE) located at Gainesville, Florida; the Invasive Insect Biocontrol and Behavior Laboratory (IIBBL) located at Beltsville, Maryland; and the Natural Products Utilization Research Unit (NPURU) located at Oxford, Mississippi. Funding for much of this research is provided by the DoD's Deployed War-fighter Protection (DWFP) Research Program, which seeks to develop insecticides with new modes of action for both military and civilian use. Discussed below are the insecticide research goals of these ARS units and the impressive results from their insecticide discovery programs.

Scientists at the MFRU are using high throughput larval screening techniques to evaluate thousands of candidate chemical compounds provided from industrial collaborators' chemical libraries. This bioassay technique exposes mosquito larvae, usually the yellow fever mosquito *Aedes aegypti*, to a large array of candidate compounds. Five first instar larvae (24 hours old) are added to each well of a 24-well plate in de-ionized water (950 ml) to which larval diet (40 ml), DMSO (dimethyl sulfoxide, a substance used to stabilize candidate chemical concentration), and the test chemical itself are added; see Figure 1. Larval mortality is scored after 24 hour post exposure. Later, the most active compounds are tested under a wide range of concentrations to determine the lethal dose necessary to kill 50 and 90% of mosquito larvae. Hundreds of chemicals can be screened weekly using this system.

Following positive hits from the

larval assay described above, candidate compounds are then tested on adult mosquitoes to determine if they will make good adulticides. Three to five day-old adult female *Ae aegypti* are anesthetized for 30 seconds with carbon dioxide and placed on a 4°C chill table. A 0.5µl droplet of pesticide diluted in acetone is then applied to the dorsal thorax using a repeating dispenser; see Figure 2. After topical treatment, mosquitoes are kept in plastic cups and supplied with 10% sucrose solution for 24 hours before mortality is scored; see Figure 3. All chemicals are subjected to a primary screen at a concentration of 200 ppm. Active compounds from the primary screen are subjected to secondary and/or tertiary screening at 20 ppm, 2 ppm, and 0.2 ppm to determine lethal dose 50 and 90 percentiles; see Figure 4. Permethrin is used as the positive control to measure relative toxicity and acetone is used as a negative control to insure that mosquito death is due to the compound and not environmental circumstances. The strategy is to pass successful adulticides to industrial collaborators for registration, production, and marketing as mosquito control insecticides. An additional inducement for industrial production and marketing is the involvement of IR-4 (Interregional Research Project Number 4), a USDA sponsored entity that facilitates the registration of minor-use pesticides. Military entomologists can then assign national stock numbers to these products through the AFPMB's pesticide committee for inclusion into military stock systems.

To date, MFRU scientists have examined over 2,000 compounds. Of these, over 200 showed high activity as a mosquito larvicide, of which 28 show high adulticidal activity. MFRU chemists are also collaborating with chemists at the University of Florida to examine the USDA historical archives of insecticide data by quantitative structure-activity relationship (QSAR) modeling to predict and synthesize new insecticides. This approach was used successfully to predict and synthesize efficacious repellents. Another source from which larvicides and adulticides can be developed is from existing insecticides already registered for use against other pests, such as the bioactive ingredients found in products registered to control other insect pests. In early 2009, 19 registered insecticides with different modes of action were tested against mosquito larvae and adults. Results indicated that 3 relatively new insecticides (ie, fipronil, spinosad, and imidacloprid) show good larvicidal and adulticidal activity, compared with that of permethrin. DWFP is now seeking product development with pesticide manufacturers through the USDA's IR-4 process to provide rapid and inexpensive registration of proven insecticides for new insect pests. For example, fipronil is the active ingredient in Maxforce® FC Roach Killer Bait Gel, a common cockroach control product used by pest control professionals in urban environments. Under the IR-4 process, mosquitoes are added as a target pest and industry then develops a product for use in the mosquito control market.

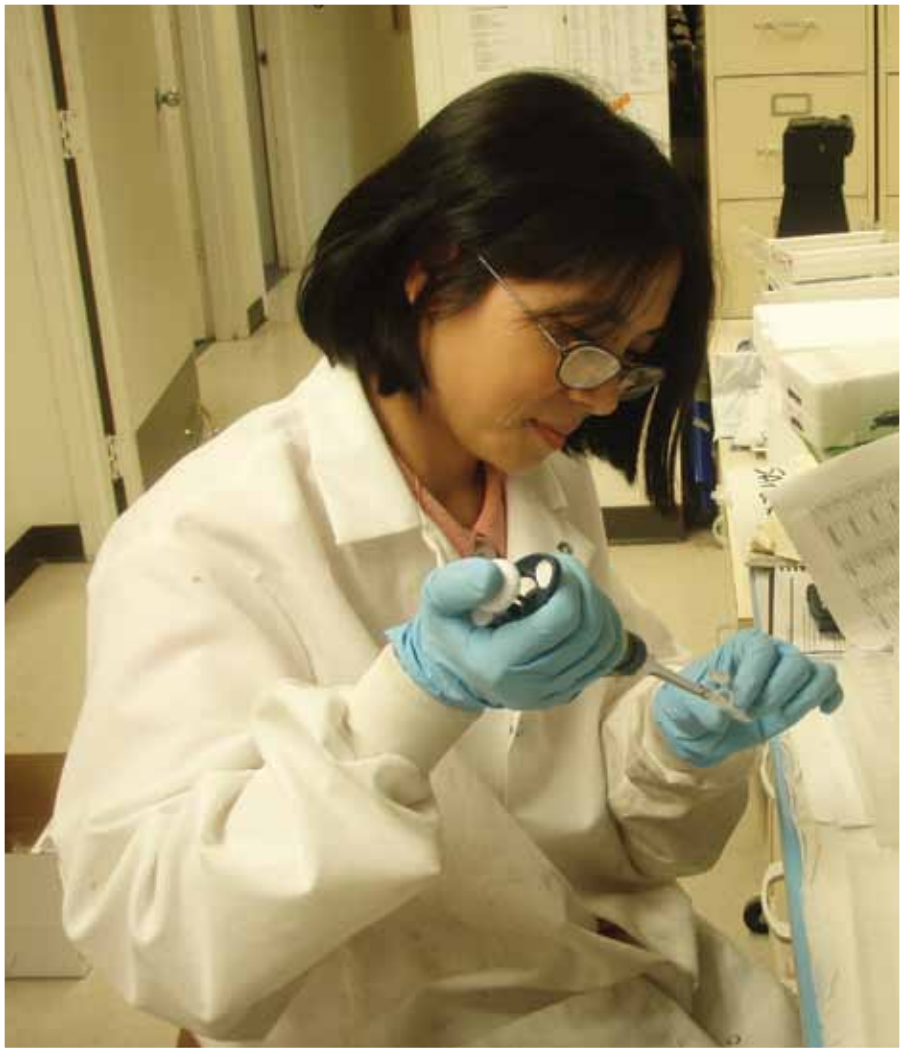

Figure 4: Dr Julia Pridgeon, CMAVE toxicologist, preparing candidate compounds for screening against *Aedes aegypti* larvae.

Figure 5: Comparison of mosquitoes surviving after 14 minute exposure to a permethrin Fire-Resistant Army Combat Uniform (FRACU) sleeve (top) and after 4 minute exposure to a FRACU sleeve treated with a fast-acting insecticide.

The Invasive Insect Biocontrol and Behavior Laboratory (IIBBL) seeks to develop insecticides from naturally-produced plant toxins. The goal of the IIBBL, funded by the DWFP, is to discover and develop public health pesticides and repellents for control of disease vectors, a key component of ARS's National Program 104 and the DWFP Program.

Development of new toxicants that provide novel modes of action or that have other properties desirable for efficacy, safety, and commercialization is based on molecules with high vapor pressure. When novel toxicants are developed, they are less susceptible to enzymatic detoxification by insects and can be developed as new, fast-acting insecticides, while insecticides with current agricultural registrations can be adapted for use against blood-sucking arthropods.

The key thrust of IIBBL's approach involves QSAR-based modeling of fast-acting pyrethroid insecticides to predict and synthesize novel compounds of a similar structure based on their known enzymatic

detoxification mechanisms in disease-carrying insects. New classes of compounds with optimized physiochemical spectrums will be then explored to develop a second generation of fast-acting insecticides. Some of these new compounds have the potential to be used on or in clothing, such as in collaborative research with MFRU scientists to find faster acting insecticides for use with military uniforms; see Figure 5. Simultaneously, IIBBL is evaluating pesticides with current agricultural registrations for their ability to control medically-important arthropods. Classes of candidate compounds obtained from existing chemical libraries and commercial sources are being screened for bioactivity via high throughput bioassays against a laboratory strain of *Ae aegypti*. Promising compounds are evaluated against additional taxa of mosquitoes and other blood-sucking arthropods including sand flies, ticks, and bed bugs, using a variety of laboratory and field bioassays. A key feature of this thrust is to evaluate laboratory toxicity in disease vectors to at least 96 hours post-exposure, since shortening the life span of a disease vector could possibly break the cycle of transmission and thereby be of great utility in vector control programs. Additionally, IIBBL scientists test, evaluate, and develop products for control on food hosts (vertebrate hosts such as cattle) and resting and breeding sites (residual and ovicidal treatments, respectively).

Research at the Natural Products Utilization Research Unit (NPURU) is concentrated in discovering natural products with insecticidal qualities from native plants. Emphasis is in finding mosquito adulticides, larvicides and repellents. NPURU scientists investigate both plant compounds and derivatives (modification of these compounds) in hopes of "enhancing" the strength of naturally active repellents and insecticides. Under the DWFP program, NPURU efforts have resulted in several potent larvicides and a repellent that is three times longer lasting and more potent than DEET.

NPURU is currently investigating terpenoid compounds common to American and Japanese Beautyberry (*Callicarpa americana* L and *C japonica* Thunb, respectively) for the repellency activity of callicarpenal and intermedeol, determined by NPURU scientists to be the responsible agents. Callicarpenal may well serve as a possible substitute for DEET, as ongoing tests with mosquitoes and ticks indicate that it is as effective as DEET and may be better tolerated by DEET-sensitive individuals.

The discovery and development of novel compounds that kill mosquitoes is a long-term and expensive endeavor, with final registration dependent upon the successful completion of a large battery of EPA-mandated tests. This testing ensures that new compounds are not only effective against mosquitoes, but that they are safe when applied to aquatic habitats or in the air and produce minimal adverse risk to human, animal, and plant health. Although the DoD-USDA ARS insecticide development program is only five years old, results obtained thus far indicate that several compounds with new modes of action have shown promise for development into future public health mosquito control products. On the horizon are new products with unique modes of action that will aid mosquito abatement and public health programs to control both larval and adult mosquitoes, especially those tolerant or resistant to older insecticides routinely used over the past 50 years.

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